available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/jprot

Resolution and identification of major peanut allergens using a combination of fluorescence two-dimensional differential gel electrophoresis, Western blotting and Q-TOF mass spectrometry

Hubert Chassaigne[a,*], *Virginie Trégoat*[a], *Jørgen V. Nørgaard*[a], *Soheila J. Maleki*[b], *Arjon J. van Hengel*[a]

[a]*Institute for Reference Materials and Measurements, European Commission-Joint Research Centre, Retieseweg 111, B-2440 Geel, Belgium*
[b]*USDA-ARS-SRRC, 1100 Robert E. Lee Boulevard, New Orleans, LA 70124, USA*

ARTICLE DATA

Article history:
Received 24 September 2008
Accepted 5 February 2009

Keywords:
Peanut allergens
Two-dimensional fluorescence gel electrophoresis
2D Western blotting
Nano-electrospray Q-TOF mass spectrometry

ABSTRACT

Peanut allergy is triggered by several proteins known as allergens. In this study, the complexity the peanut allergome is investigated with proteomic tools. The strength of this investigation resides in combining the high-resolving power and reproducibility of fluorescence two-dimensional differential gel electrophoresis with specific immunological detection as well as polypeptide sequencing by high-resolution mass spectrometry. Matching of the peanut proteins in 2D gels was achieved by differential labelling whereby peanut proteins and purified allergens (Ara h 1, Ara h 2 or Ara h 3/4) were run on the same gel. Ten protein spots on a mass line of ca. 63–68 kDa were likely to correspond to Ara h 1. Two doublets on two different mass lines at ca. 16 and 18 kDa matched with purified allergen Ara h 2. The basic and acidic sub-units of Ara h 3/4 were observed at masses of ca. 25 kDa and 40–45 kDa, respectively. Subsequently the antibody-binding capacity of spots corresponding to peanut allergens was investigated by Western blotting of 2D gels using antibodies (IgY) raised against Ara h 1, Ara h 2 and the recombinant 40 kDa sub-unit of Ara h 3/4. Final confirmation of the identity of the protein spots matched after 2D electrophoresis and identified by Western blotting was obtained by in-gel digestion of protein spots and analysis by quadrupole time-of-flight mass spectrometry. By using the method developed in our work, the location and identification of two different isoforms of the allergen Ara h 1, the allergen Ara h 2 and six isoforms of the allergen Ara h 3/4 in 2D peanut protein maps was established.

1. Introduction

Food allergens are a significant worldwide public health issue. Estimates for the prevalence of food allergies are around 1–2% of the total population and up to 8% of children, although the prevalence may vary between populations and age groups [1–3]. The severity of allergic reactions can vary from symptoms ranging from mild urticaria to potentially lethal anaphylactic shock. Over recent years legislation has been issued aimed at a better safeguarding of the health of allergic consumers. New food allergen labelling rules were introduced by European Directives 2003/89/EC and 2007/68/EC, two successive amendments to the general food labelling directive 2000/13/EC of the European Parliament and of the Council [4–6]. The legislation requires food manufacturers to indicate 14 groups of potential allergens by reference to the source allergen if they are used as

* *Corresponding author*. Tel.: +32 14 57 12 56; fax: +32 14 57 17 87.
E-mail address: hubert.chassaigne@ec.europa.eu (H. Chassaigne).

1874-3919/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jprot.2009.02.002

an ingredient at any level in pre-packed foods. Peanuts are among the most common causes of immediate hypersensitivity reactions to foods [7,8] and therefore required to be declared on food labels.

Usually food allergens, the components capable of triggering allergic reactions, are proteins, but only a small percentage of proteins in food are allergenic. The allergome of peanut is rather complex. The three major peanut allergens that are recognised by the vast majority of peanut allergic individuals and that have been studied are Ara h 1, a vicilin-like protein [9,10], Ara h 2, a conglutin–homologue protein [9,11] and Ara h 3/Ara h 4, glycinin proteins [12–14]. Recently it was shown that Ara h 3 and Ara h 4 are isoallergens and can be designated as the allergen Ara h 3/4 [15,16]. Peanuts contain an average of 29% protein. The allergen Ara h 1 is the most abundant (20% of the total protein) followed by Ara h 2 (~10%) and Ara h 3/4 [17,18].

The properties of those main peanut allergens have been studied in recent years. Ara h 1 is a 63–68 kDa glycoprotein [19] assembled in di- and trimeric complexes [20,21] and two of its genes encoding 626 and 614 amino acids have been well described [22,23]. Of the allergen Ara h 2 two isoforms with masses of ca. 16 and 17 kDa have been isolated [24,25]. The allergenic protein Ara h 3/4, recently purified consists of an acidic and a basic sub-unit [14,26,27]. The two sub-units remain covalently linked by an intermolecular disulfide bridge and associate into a very stable hexameric structure [16,28]. The acidic sub-unit has a molecular mass in the range of 40–45 kDa, whereas the basic sub-unit has a mass of ca. 25 kDa. Ara h 3/4 is mainly proteolytically modified (truncation at multiple sites), with possible glycosylation. Proteolytic truncation was observed for the acidic sub-unit but not for the basic sub-unit resulting in a series of polypeptides ranging from 13–45 kDa [14].

A proper assessment of temperature stability as well as qualitative or quantitative analysis of individual peanut allergens requires a separation of their different isoforms/glycoforms, protein sub-units and possible proteolytic products with high-resolution techniques. For this reason, our research will focus on the characterisation of the peanut allergome based on a 2D gel electrophoresis (2D PAGE) approach. This approach is a combination of three different techniques. Individual techniques have a series of advantages and limitations and their combined use can reduce those limitations.

2D PAGE utilises a few milligrams of protein and separates them into thousands of protein spots [29,30]. Comparison of gel images between different peanut extracts or between peanut proteins and purified allergens can be achieved by 2D PAGE [31]. Fluorescence two-dimensional gel electrophoresis (2D DIGE) represents an elegant modification of 2D PAGE eliminating gel-to-gel variation in protein migration that hinder computer-assisted comparison of spot patterns [32]. After 2D DIGE the allergen protein spots need to be specifically detected and identified. This can be achieved by a combination of western blotting of 2D protein gels allowing the detection of antibody-specific reactive proteins, and in-gel digestion of protein spots followed by mass spectrometry [33]. In this work, we have applied 2D DIGE in combination with Western blotting and high-resolution mass spectrometry for the separation, immunodetection and identification of the major peanut allergens Ara h 1-3/4 in 2D peanut protein patterns. The latest generation of proteomics instruments includes the quadrupole time-of-flight (Q-TOF) tandem mass spectrometer. The major advantages of the Q-TOF instrument are ultra-high throughput, high sensitivity, and high-resolution capabilities that provide enhanced peptide-sequence information.

2. Materials and methods

2.1. Chemicals

All chemicals used for sample preparation were obtained from VWR International (West Chester, PA, USA) and were at least analytical reagent grade. Water from a milli-Q water system (Millipore, Bedford, MA, USA) was used throughout. PlusOne chemicals for gel electrophoresis (Tris, glycine, CHAPS, urea, thiourea, DTT, dimethylformamide), CyDyes (Cy3 and Cy5), acrylamide, bis-acrylamide, Cy3-antibodies labelling kit, 2D Clean-up and 2D Quantification kits were purchased from GE Healthcare (Uppsala, Sweden). Ampholytes and immobilised pH gradient strips were supplied by Bio-Rad Laboratories (Hercules, CA, USA). Mini-gels for electrophoresis were from Invitrogen (Carlsbad, CA, USA). Secondary antibodies (non-conjugated rabbit anti-chicken IgY (whole molecule)) and bovine caseins were from Sigma-Aldrich (St Louis, MO, USA). Sequencing grade trypsin was obtained from Merck (Darmstadt, Germany). SupraPure Formic acid and HPLC grade acetonitrile for mass spectrometry were from Sigma-Aldrich (St Louis, MO, USA).

2.2. Materials

Peanut samples (*Arachis hypogaea*) of the variety Chinese Virginia were obtained from IMKO (Doetinchem, The Netherlands). This peanut variety is included in the IRMM-481 test material. The peanuts were blanched to remove the skins. This processing was done by the supplier following standard industrial processing technology.

Purified allergens Ara h 1, Ara h 2 and Ara h 3/4 were isolated from crude peanut extracts as previously described [8,26,34]. The anti-Ara h 1, Ara h 2 and Ara h 3/4 antibodies used in the Western blot analysis were chicken egg yolk polyclonal antibodies (IgY) custom manufactured by Sigma Immunosys (The Woodlands, TX, USA).

2.3. Sample preparation and protein labelling

Peanuts were ground under liquid nitrogen in order to obtain a fine and homogeneous powder. Extraction was performed at 4 °C using TBS buffer (20 mM Tris with 150 mM NaCl) pH 7.4 according to Chassaigne *et al.* [31]. Peanut extracts and purified peanut allergens were cleaned with the 2D Clean up kit before the determination of their protein content with the 2D Quant kit from GE Healthcare (Uppsala, Sweden).

Protein aliquots in the range 5–25 μg (peanut protein or peanut allergen) were solubilised in the DIGE labelling buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris, pH adjusted to 8.9 with HCl 1 M) at a final concentration of 5 μg/μl. Each dye (Cy3 and Cy5) was reconstituted in dimethylformamide to a final concentration of

1 nmol/μl. This stock solution was diluted (200 pmol/μl) immediately prior to use and 0.5 μl Cy3 was added/25 μg peanut protein or 0.1 μl Cy5/5 μg purified peanut allergen. Following incubation for 30 min on ice in the dark, the reactions were quenched by adding 0.5 μl 10 mM lysine solution, followed by further 10 min incubation on ice in the dark. Protein samples previously labelled with the respective fluorescent dyes (Cy3 and Cy5) were used separately or pooled and diluted in the IPG (immobilised pH gradient) rehydration buffer (8 M urea, 0.5% CHAPS, 0.5% IPG buffer pH 3–10, 0.002% bromophenol blue, 0.4% DTT) for isoelectric focusing (IEF).

2.4. 2D electrophoresis of peanut proteins and purified allergens

First-dimension IEF was carried out on an Ettan IPGphor system (GE Healthcare). Twenty five μg Cy3-labelled peanut protein and 5 μg Cy5-labelled purified allergen were pooled and diluted in a total volume of 240 μl IPG rehydration buffer. The sample was loaded on 24 cm pre-cast immobilised pH gradient strips (240×3.0×0.5 mm, linear pH range: 3–10). IPG strips were rehydrated for 12 h at 20 °C. IEF was performed for 53500 Vh with the initial voltage set to 500 V for 1 h, and then stepped up to 1000 V for 1 h and finally to 8000 V for 6.5 h. The gel strips were prepared for transfer to the second dimension by soaking for 15 min in an equilibration solution (50 mM Tris–HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue and 10 mM DTT). The equilibrated gel strips were embedded at the top of the SDS-PAGE gel in molten 1% (w/v) agarose in cathodic electrode buffer (Laemmli buffers).

Second dimension separation (SDS-PAGE) was carried out with an Ettan DALT electrophoresis unit equipped with a Peltier cooling unit. Gels were cast in fluorescent-compatible quartz cassettes (260×200×1.0 mm, homogeneous 12.5%, separation range 10–100 kDa) and SDS-buffers (Laemmli buffers) were used. Six gels were run at 5 W/gel for 15 min (30 W), then 20 W/gel for 4 h (120 W) with the Peltier cooling system at 25 °C. Each gel was made in triplicate.

After the electrophoretic separation, the 2D gels still contained between the quartz plates were scanned with a Typhoon 9400 series variable mode imager system (GE Healthcare). The fluorescently labelled proteins were visualised with the following wavelengths: Cy3–532 nm excitation wavelength, 580 BP 30 emission filter and Cy5–633 nm excitation wavelength, 670 BP 30 emission filter. All gels were scanned at 100 μm resolution.

After the images were acquired, Image Master 2D Platinium 6.0 software (GE Healthcare) was used for protein detection, matching and exporting data. The fully automated co-detection algorithm ensured that all spots on co-detected gel images (Cy3 and Cy5 channels) were represented identically and were overlaid. The apparent molecular mass of proteins was determined by co-electrophoresis of protein markers (MW range 14–100 kDa) (GE Healthcare). The pI values were evaluated according to the scale of the linear immobilised pH gradient strips (pH range 3–10).

2.5. 2D PAGE and Western blotting

To perform 2D electrophoresis experiments combined with Western blotting 15 μg Cy3-labelled peanut protein diluted in 120 μl IPG rehydration buffer was loaded on a 7 cm IPG strip pH 3–10, linear range (70×3.0×0.5 mm) (GE Healthcare). Separation in the first dimension was performed with an initial voltage at 320 V for 1 h, then a gradient up to 1000 V for 30 min, then a gradient up to 5000 V in 1 h 20, and finally stepped to 5000 V for 30 min. A XCell SureLock Mini-Cell electrophoresis system (Invitrogen) and a Power Pac 200 power supply (Bio-Rad Laboratories) were used for the second dimension. The equilibrated 7 cm IPG gel strip was embedded at the top of a pre-cast NuPAGE Bis–Tris SDS-PAGE 12% (80×70×1.0 mm) mini-gel (Invitrogen). Protein separation was carried out under a constant voltage of 150 V for 1.5 h in NuPAGE MOPS SDS running buffer (2.5 mM MOPS, 2.5 mM Tris Base, 0.005% SDS, 0.05 mM EDTA, pH 7.7).

Each experiment was made in duplicate. Mini-gels were removed from the cassettes and placed between quartz plates for scanning. Scanning was performed with the Typhoon 9400 system at 50 μm resolution both before and after Western blotting to check transfer efficiency. The wavelength settings of Cy3 detection are described in Section 2.4.

The gels were equilibrated in diluted NuPAGE transfer buffer (20×) (25 mM Bis–Tris, 1.025 mM EDTA, 25 mM Bicine, pH 7.2) before proteins were transferred by electroblotting to PVDF membranes (GE Healthcare) that had been pre-wetted with methanol, rinsed in milli-Q water and incubated for a few minutes in transfer buffer. Blots were blocked with 3% caseins (bovine) in Tris-buffered saline (TBS buffer) pH 7.4, 0.1% Tween 20 for either 1 h at room temperature or overnight at 4 °C under gentle agitation. The membranes were rinsed 3 times 10 min with TBS buffer pH 7.4, 0.5% Tween 20 and subsequently incubated with primary antibodies (IgY raised against Ara h 1, Ara h 2 or a 40 kDa sub-unit of Ara h 3/4) used at a dilution of 1:10,000 in TBS buffer pH 7.4, 0.1% Tween 20, 3% casein for 1 h at room temperature under constant shaking. After 3 washes of 10 min with TBS buffer pH 7.4, 0.5% Tween 20, the membranes were incubated for 1 h at room temperature with secondary antibodies (rabbit anti-chicken IgY labelled with Cy5 as described in Section 2.3) and visualised with the wavelength settings of Cy5 described in Section 2.4 after a final series of 3 times 10 min washes. Using simultaneously the wavelength settings for Cy3 and Cy5 (Cy5 channel for antibody-reactive proteins and Cy3 channel for total protein) ensured that spots present on co-detected membrane images were perfectly overlaid.

2.6. Preparative 2D gels and mass spectrometry

500 μg unlabelled-peanut protein was loaded on a 24 cm IPG strip (pH 3–10) for preparative experiments. Experimental conditions for electrophoresis were the same as the ones described for analytical gel in Section 2.4. For the preparative workflow, glass plates instead of quartz plates were used for gel casting. A silane binding treatment of the glass plates (80% ethanol, 2% acetic acid, 0.1% silane binding) was performed to immobilise the gels onto the glass plates. Two white stickers used as reference for x, y coordinates were attached on the treated surface of the plate to ensure that the correct protein spots detected were picked. Each gel was made in triplicate.

The 2D gel cassettes were opened and the gels for spot picking were stained using Deep purple total protein stain according to the manufacturer's protocol (GE Healthcare). The fluorescently labelled proteins were visualised with the 2D gel still attached to the treated glass plate of the cassettes using the Typhoon 6400 system with the wavelength settings of Deep purple (532 nm excitation wavelength, 560 nm LP emission filter). Selected protein spots were excised and transferred to a 96-well plate using the Ettan Spot Picker system (GE Healthcare).

The spots were de-stained (200 mM ammonium bicarbonate), washed with CH_3CN:H_2O, 50:50, reduced (using 10 mM DTT in 50 mM ammonium bicarbonate, 30 min) and alkylated (using 55 mM iodoacetamide in 50 mM ammonium bicarbonate, 20 min). The enzymatic digestion (using 1 ng/μl sequencing grade trypsin in 100 mM ammonium bicarbonate) was performed at 37 °C for 5 h. In the case of Ara h 2 spots, a different enzymatic treatment was applied. RapiGest (Waters) was added to the 50 mM ammonium bicarbonate solution during the reduction step (using DTT). Enzymatic hydrolysis used 1 ng/μl pancreatin in 50 mM ammonium bicarbonate and was performed at 50 °C for 5 h. The resulting hydrolysates were extracted in a total volume of 60 μl solution (1% HCOOH in CH_3CN:H_2O, 2:98) and transferred into 96-well PCR plates.

LC-MS/MS analyses were performed on a capillary flow-liquid chromatography system (CapLC) coupled on-line with a quadrupole time-of-flight mass spectrometer (Q-TOF Ultima Global) equipped with a nano-electrospray source (Waters, Manchester, UK). 96-well plates containing protein hydrolysates were loaded on the autosampler of the capillary LC system. A pre-column Acurate micro-flow splitting system (LC Packings, Sunnyvale, CA, USA) was used. The binary CapLC pump was operated at a flow rate of 10 μl/min and a flow rate of 1 μl/min was obtained after a 1:10 split. Peptide mixtures were desalted and pre-concentrated on LC Packings C_{18} capillary cartridges (300 μm id, 150 mm length, 5 μm particles size) and separated on a Grace Vydac C_{18} High-loading capacity capillary column (150 μm id, 150 mm length, 3 μm particles size, 190 Å porosity). The Q-TOF instrument was operated in the “MS survey” mode, which means that a fragmentation of ions was achieved when a minimum intensity (specified value) was detected (3 channels for a simultaneously fragmentation of the 3 most abundant precursor ions). Experimental conditions for CapLC and Q-TOF were described previously [35].

2.7. Protein database searching parameters

The fragment ion spectra obtained from the “MS survey” mode were processed using Mass Lynx version 4.0 (Waters), a software program that converts MS/MS raw data to peak lists [35]. After centroiding and background subtraction, the peak lists were used to search databases with Protein Lynx Global Server 2.2.5 (Waters). Proteins were identified by correlation of processed tandem mass spectra to protein entries. The protein sequence database Swiss-Prot and a reduced database of known peanut allergens were used. The latter contained: Allergen Ara h 1, clone P41B [Precursor] (Swiss-Prot entry P43238); Allergen Ara h 1, clone P17B [Precursor] (P43237); Allergen Ara h 2.01 (Q941R0); Allergen Ara h 2.02 (Q6PSU2); Allergen Ara h 3 Glycinin [Fragment] (O82580); Glycinin [Fragment] (Q6IWG5); Gly 1 (Q9F7Z11); Allergen Ara h 3/Ara h 4 (Q8LKN1); Allergen Ara h 4 (Q9SQH7); and allergen iso-Ara h 3 (Q0GM57).

Protein Lynx Global Server 2.2.5 submitted the peak lists to a workflow: i) databank searching and ii) the “auto-mode” extended tool. Maximum 3 missed cleavages per peptide were allowed. Precursor ion tolerance window and fragment ion tolerance mass were set to 100 ppm and 0.05 Da, respectively. Tolerance on mass calibration of the TOF was set to 30 ppm. Carboxymethylation of cysteine and oxidation of methionine residues were assumed (fixed modifications), but other potential modifications were also considered (variable modification). Search results were validated when at least 3 consecutive measured fragment ions of a peptide matched theoretical b- or y- fragment ions of a known protein sequence tag.

After databank searching and sequence analysis each identified peptide was assigned a ladder score (number of consecutive or complementary b- and y- fragment ions) and a probability based score algorithm, which gave an indication for the reliability of the peptide identification. The peptide identification is validated by the accuracy of peptide fragments and the quality of the mass spectrum. The method employs a discriminant function with classified “good” and “bad” spectra, producing a single quality peptide score. The classification of “good” spectra starts from a peptide score of 20 at a significance level of $P<0.05$ (interval of confidence of 95%) for the individual peptides. Only peptides fulfilling all conditions listed above are validated. In any case, three MS/MS runs are made for each sample and only peptides found in the three replicate runs are accepted as a confident identification [35].

3. Results and discussion

3.1. Analysis of 2D gels of peanut protein spiked with purified allergens

To identify the position of the major peanut allergens in 2D protein maps, raw peanut extracts and purified allergenic proteins were used. Proteins from raw peanut and a purified allergen (either Ara h 1, Ara h 2 or Ara h 3/4) were labelled with two distinct fluorescent dyes and run in the same 2D gel. To exclude artefacts caused by electrophoresis conditions, 2D gels were run in triplicate. From each gel, two images were obtained: a Cy3 image for raw peanut extract and a Cy5 image for the purified allergen. Typically three to four hundreds of spots were detected in the peanut protein map. For our application to protein detection and identification, the maximum number of spots to match between the Cy3 and Cy5 images was set to two hundreds.

Panel a in Fig. 1 shows the gel map of proteins from the raw peanut extract. The inserts delimited by dashed lines show peanut protein spots matching purified allergens to which numbers were attributed. Panel b in Fig. 1 shows a part of the gel image obtained for the purified allergen Ara h 1. Spots 1–10 on a mass line of ca. 63–68 kDa and covering a pI range of 6.5–7 are likely to correspond to different glycosylation forms and/or isoforms of Ara h 1. This is in agreement with the results reported by Liang *et al.* [36] who separated the 65 kDa Ara h 1 into many spots with different pIs by 2D PAGE. Several intense spots observed within the sample of purified Ara h 1 found on a lower mass line do not match the one of peanut proteins (Fig. 1b) and therefore might contain Ara h 1 degraded *in planta*, or fragmented during purification.

Panel c in Fig. 1 shows a part of the gel image corresponding to the purified allergen Ara h 2. Four intense spots, on two different mass lines (ca. 17 and 19 kDa) and two different pIs (ca. 5.8 and 6.2) were matched with the purified allergen Ara h 2 (spot numbers 19–22). Those spots seem to coincide with the two isoforms of Ara h 2 available in the Swiss-Prot database with theoretical masses of ca. 16 and 18 kDa that encode 135 and 151 amino acids (difference of 16 amino acids). Burks *et al.* [37,38] showed that the N-terminal amino acid composition of the two bands obtained by 1D SDS-PAGE at 17 kDa and 19 kDa was essentially identical and that Ara h 2 isoforms are highly glycosylated (20% carbohydrate) with significant levels of galacturonic acid. We assume that the two spots on the two different mass lines (19, 20 at ca. 19 kDa on one side and 21, 22 at ca. 17 kDa on the other side in Fig. 1 Panel c) are likely to correspond to two isoforms of Ara h 2 and that the pI differences between spots 19 and 20 as well as between spots 21 and 22 might be a result of carbohydrate composition.

The 2D pattern of the purified Ara h 3/4 appears more complex (Fig. 1d) and the matching led to the establishment of a

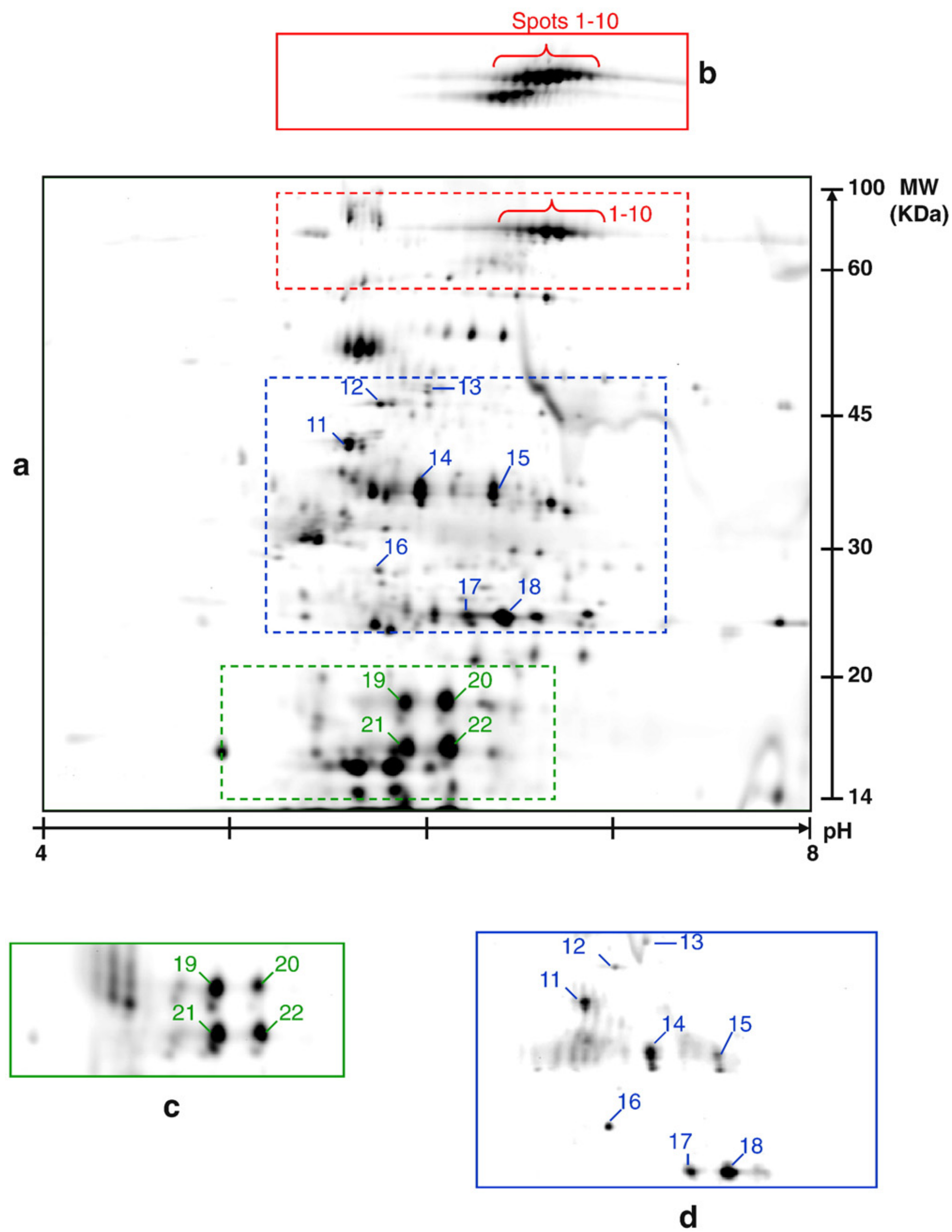

Fig. 1 – 2D DIGE images of peanut proteins and the purified allergens Ara h 1–3. Peanut protein and a purified allergen (Ara h 1, Ara h 2 or Ara h 3) were mixed and separated in the same SDS-PAGE gel. a) Annotated gel map of protein from peanut extract (Cy3 image). The inserts delimited by dashed lines show peanut protein spots matching the purified allergens (Cy5-labeled); matched spots with purified allergens were numbered (1–22). b) spots of the purified allergen Ara h 1 (Cy5 image); spot numbers 1–10. c) spots of the purified allergen Ara h 2 (Cy5 image); spot numbers 19–22. d) spots of the purified allergen Ara h 3 (Cy5 image); spot numbers 11–18.

list of 8 spots (numbers 11–18). In contrast to recombinant Ara h 3/4, a 60 kDa single-chain polypeptide, Piersma *et al.* [14] showed that the allergen isolated from its native source is extensively proteolytically processed. In addition to the acidic and basic sub-units, several proteolytic products were observed for the acidic sub-unit. Besides the major bands at 45, 40 and 25 kDa a series of Ara h 3/4 polypeptides ranging from 12–35 kDa was separated by 1D SDS-PAGE [14]. This was confirmed by Koppelman et al [26] who highlighted by 1D SDS-PAGE abundant proteins of the acidic sub-unit (42 and 45 kDa) and the basic sub-unit (25 kDa) of Ara h 3/4 besides some proteolytic fragments at 14 kDa. In this work, spots 11–13 were found to have a mass in the range of 40–45 kDa whereas spots 17 and 18 correspond to an apparent mass of 25 kDa. Spots 14, 15 (ca. 35 kDa) and 16 (ca. 30 kDa) perfectly matched purified allergen Ara h 3/4 and therefore might be proteolytic products of Ara h 3/4.

Interpretation of the above reported results is strongly linked to the nature (eg. different peanut variety) and level of purity of the samples. Additionally, the purification process does not likely yield all isoforms or glycoforms of the protein. The identified spots were therefore further characterised by immunoblotting and mass spectrometric techniques.

3.2. *Peanut allergen detection by Western blotting analysis*

After 2D electrophoresis the allergen protein spots were identified by Western blotting, which represents a powerful technique to reveal the position of antibody-reactive proteins

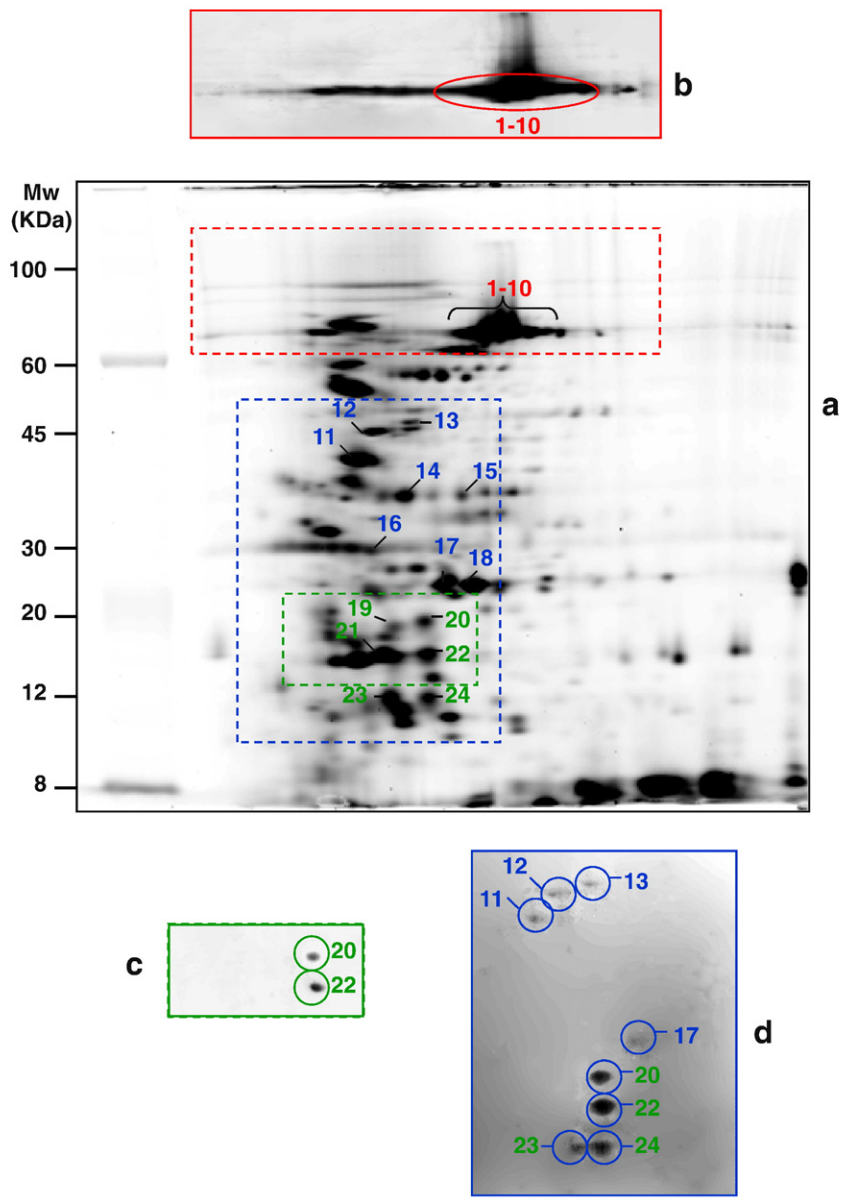

Fig. 2 – Detection of the major peanut allergens by Western blotting analysis. Peanut proteins were labelled with Cy3. Antibodies against: i) the native form of Ara h 1, ii) the native form of Ara h 2, and iii) a recombinant 40 kDa sub-unit of Ara h 3 were used. Detection was done by using secondary antibodies labelled with Cy5. a) Cy3 image of protein gel from peanut protein extract before transfer to the membrane; spot numbers of matched spots found in Fig. 1 are shown. b) Cy5 image of the Western blot of anti-Ara h 1. c) Cy5 image of the Western blot of anti-Ara h 2. d) Cy5 image of the Western blot of anti-Ara h 3.

within 2D gel maps. Following 2D separation and transfer on PVDF membrane, the peanut proteins were immunodetected with chicken yolk antibodies (IgY) raised against Ara h 1, Ara h 2 and a recombinant 40 kDa sub-unit of Ara h 3/4. The gels and Western blots were performed in duplicate.

From panel a in Fig. 2 it is evident that the 2D pattern of Cy3-labelled peanut proteins is quite rich in well separated proteins even though a lower resolution was obtained using the mini-gels in comparison to analytical gels (Fig. 1). The inserts delimited by dashed or continuous lines show antibody-reactive peanut protein spots to which numbers were attributed. Protein spots were numbered as in Fig. 1. Efficiency of protein transfer was controlled by scanning the membrane (data not shown). An efficient transfer of proteins was obtained even though the relative proportion of some spots was modified especially for the lower masses (below 30 kDa).

Anti-Ara h 1 identified a closely migrating series of spots with a mean molecular mass of 63–68 kDa, covering a pI range of 6.5–7 as shown (panel b in Fig. 2). This was similar to that seen for purified Ara h 1 in Fig. 1a. Spots 1–10 are likely to correspond to the two different isoforms and multiple glycoforms of Ara h 1. This suggests that both isoforms of Ara h 1 are antibody-reactive proteins and that glycosylation does not significantly affect antibody binding.

Using the antibody raised against the purified allergen Ara h 2 identified two proteins of 17 and 19 kDa as shown in panel c in Fig. 2 (spot numbers 20 and 22 as in Fig. 1c). This implies that both Ara h 2 isoforms are detected but that glycosylation affects antibody binding since spots 19 and 21 were not recognised with anti-Ara h 2 (panel c in Fig. 2).

Compared to the eights spots matching purified allergen when gels were spiked with Ara h 3/4 (numbers 11–18 in Fig. 1) the IgY antibody raised against the recombinant 40 kDa sub-unit of Ara h 3/4 only recognised three of those spots (numbers 11–13, panel d in Fig. 2). These spots at ca. 40–45 kDa and within a pI range of 5.5–6 therefore contain the acidic sub-unit of the allergen Ara h 3/4 as described in the literature [14]. Spots 14–16 and 18 were not detected by immunoblotting and therefore either do not contain the acidic sub-unit of Ara h 3/4 or lack IgY epitopes due to proteolytic processing or sequence heterogeneity amongst Ara h 3/4 isoforms. Piersma *et al.* [14] showed that the acidic sub-unit of Ara h 3/4 is proteolytically truncated at multiple sites, whereas no processing of the basic sub-unit was observed. Spot 17 at ca. 25 kDa and a pI of 6.3 was also recognised with a very low intensity, and although this molecular mass corresponds to that of the basic sub-unit of Ara h 3/4 (Fig. 2d), at this stage it is unlikely that this spot corresponds to the basic sub-unit since the antibody used was raised against the 40 kDa acidic sub-unit of Ara h 3/4. The two spots 23 and 24 (observed masses of ca. 12 kDa) were also recognised by the antibody (Fig. 2d) and are likely to correspond to proteolytic fragments of Ara h 3/4 as described by Koppelman *et al.* [14]. Surprisingly, spots 20 and 22, likely to correspond to one isoform of Ara h 2, were also recognised by anti-Ara h 3/4. This might be due to common IgY binding epitopes since the two major peanut allergens are know to share homologous IgE epitopes [39].

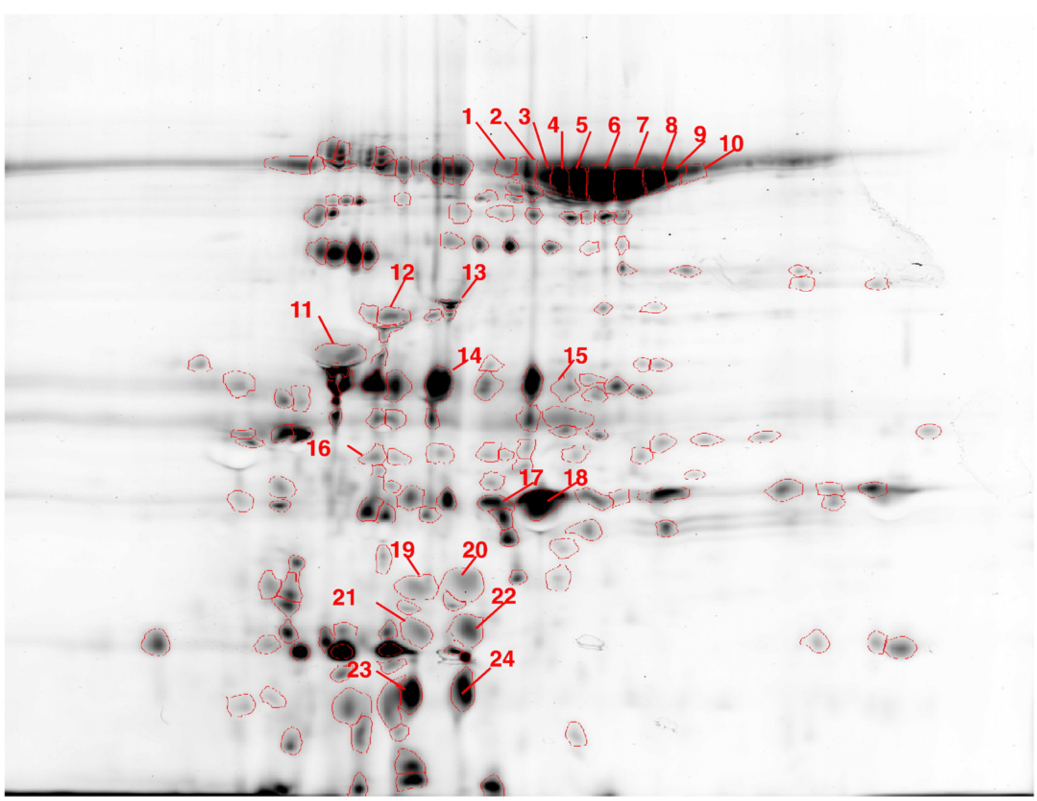

Fig. 3 – Annotated reference 2D map of peanut protein for identification by Q-TOF MS/MS. 500 μg non-labelled protein from raw peanut was run on the 2D gel. Spots of interest selected from Deep purple total protein stain were excised, submitted to tryptic digestion and analysed by MS/MS. Each selected spot is pointed with a red arrow and corresponding spot number (1–24) is specified. The identities of some selected protein spots are presented in Tables 1–3.

Table 1 – Validation of identified Ara h 1, Ara h 2 and Ara h 3/4 protein spots by Q-TOF mass spectrometry.

Protein spot number	Protein name	Source and accession number	Protein probability (%)	Number of peptides identified
1	Ara h 1 clone P17	Swiss-Prot, P43237	54	6
	Ara h 1 clone P41B	Swiss-Prot, P43238	51	6
2	Ara h 1 clone P41B	Swiss-Prot, P43238	100	6
3	Ara h 1 clone P17	Swiss-Prot, P43237	100	12
4	Ara h 1 clone P41B	Swiss-Prot, P43238	100	15
5	Ara h 1 clone P17	Swiss-Prot, P43237	100	15
	Ara h 1 clone P41B	Swiss-Prot, P43238	100	20
6	Ara h 1 clone P17	Swiss-Prot, P43237	100	12
	Ara h 1 clone P41B	Swiss-Prot, P43238	100	18
7	Ara h 1 clone P17	Swiss-Prot, P43237	100	17
	Ara h 1 P41B	Swiss-Prot, P43238	100	21
8	Ara h 1 clone P41B	Swiss-Prot, P43238	100	6
9	Ara h 1 clone P41B	Swiss-Prot, P43238	100	9
10	Ara h1 clone P17	Swiss-Prot, P43237	59	7
	Ara h1 clone P41B	Swiss-Prot, P43238	46	7
11	Ara h 3 Glycinin	Swiss-Prot, O82580	100	4
	Gly 1	Swiss-Prot, Q9FZ11	20	1
	Ara h 4	Swiss-Prot, Q9SQH7	25	2
	Allergen Ara h 3/Ara h 4	Swiss-Prot, Q8LKN1	96	2
	Glycinin	Swiss-Prot, Q6IWG5	50	2
	Iso-Ara h 3	Swiss-Prot, Q0GM57	25	2
12	Ara h 3 Glycinin	Swiss-Prot, O82580	50	4
	Gly 1	Swiss-Prot, Q9FZ11	100	4
	Ara h 4	Swiss-Prot, Q9SQH7	20	1
	Allergen Ara h 3/Ara h 4	Swiss-Prot, Q8LKN1	25	2
	Glycinin	Swiss-Prot, Q6IWG5	20	1
	Iso-Ara h 3	Swiss-Prot, Q0GM57	20	1
13	Ara h 3 Glycinin	Swiss-Prot, O82580	36	3
	Gly 1	Swiss-Prot, Q6IWG5	20	1
	Ara h 4	Swiss-Prot, Q9FZ11	25	2
	Allergen Ara h 3/Ara h 4	Swiss-Prot, Q0GM57	50	4
	Glycinin	Swiss-Prot, Q8LKN1	20	1
	Iso-Ara h 3	Swiss-Prot, Q9SQH7	20	1
17	Ara h 3 Glycinin	Swiss-Prot, O82580	20	1
	Gly 1	Swiss-Prot, Q6IWG5	20	1
	Ara h 4	Swiss-Prot, Q9FZ11	20	1
	Allergen Ara h 3/Ara h 4	Swiss-Prot, Q0GM57	25	2
	Glycinin	Swiss-Prot, Q8LKN1	20	1
	Iso-Ara h 3	Swiss-Prot, Q9SQH7	20	1
20	Ara h 2.01	Swiss-Prot, Q941R0	53	2
	Ara h 2.02	Swiss-Prot, Q6PSU	52	2
22	Ara h 2.01	Swiss-Prot, Q941R0	51	2
	Ara h 2.02	Swiss-Prot, Q6PSU	54	2

The table shows the name and Swiss-Prot accession number of proteins identified in individual spots of 2D gels of peanut protein extracts (spots numbered 1–22 in Figs. 1–3). Each protein has been assigned a probability score and the number of peptides identified for a specific isoform is shown.

In this study, IgY antibodies raised against the purified allergens allowed their localisation in the 2D protein map. However, additional investigations by mass spectrometry were required to unambiguously confirm the identity of the peanut protein spots that matched the position of the purified allergens in the 2D map and showed antibody-reactivity.

3.3. *Confirmation of the identified proteins by capillary LC and Q-TOF MS/MS*

Final confirmation of the identity of protein spots in 2D gel maps was obtained by capillary LC and Q-TOF MS/MS analysis. Protein spots matched after DIGE analysis of peanut protein

Notes to Table 2:
The table shows Ara h 1 derived peptides identified in individual spots of 2D gels of peanut protein extracts (spots numbered 1–10 in Figs. 1–3). Reported sequences were Ara h 1 clone P17 and Ara h 1 clone P41B (source: Swiss-Prot, entry numbers P43237 and P43238). Each peptide is given with the corresponding ladder score which indicates the reliability of the peptide identification. Peptides on a dark grey background are specific for one of the two isoforms of Ara h 1. a) Double charged peptide unless otherwise indicated (+, +++ or ++++). b) Peptide ladder score: probability based score algorithm, which indicates the reliability of the peptide identification.

Table 2 – List of identified peptides derived from the peanut allergen Ara h 1.

Protein Name: **Allergen Ara h 1 clone P41B** [Precursor]	Protein MW (Da): 71302, pI: 7.02	Source and accession number: SwissProt/TrEMBL, P43238 isofom 1
Protein Name: **Allergen Ara h 1 clone P17** [Precursor]	Protein MW (Da): 71302, pI: 7.02	Source and accession number: SwissProt/TrEMBL, P43237 isoform 2

Isoform 1	Isoform 2	Peptide sequence	Substitution	Submitted mass (Da)[a]	**Peptide ladder score (%)[b]	Spot number in gel
X		(V)VASISATHAKSSPYQKKTENPC(A)	V for L (1), I for V (4)	850.732+++	22	5
	X	(L)ASVSATQAKSPYRKT(E)		726.324+++	17	1
	X	(R)WGPAEPR(E)		406.717	74	5
	X	(R)EGEQEWGTPGSEVR(E)		780,876	62	6, 7
X		(R)EGEQEWGTPGSHVR(E)		523.592	25	7
X	X	(R)NNPFYFPSRR(F)		433.218+++	39	4,5,12
X	X	(R)QFQNLQNHR(I)		592.812	71	3,4,5,6,7
X	X	(R)FQNLQNHR(I)		395.207+++	40	3,5,6,7
	X	(R)IVQIEAR(P)		414.740	87	3,5,7,10
X		(R)IVQIEAKPNTL(V)		613.347	78	4,5,6,7
X		(R)IVQIEAKPNTLVLPK(H)		555.008+++	56	2
X	X	(D)NIMIIQ(Q)	M for L (3), I for V (4)	759.356+	72	4,10
X	X	(N)RKSWNLDEGHAVR(I)	W for F (4), V for L (12)	449.213++++	39	5
X	X	(K)SFNLDEGHALR(I)		420.202+++	45	7
X	X	(Q)NLRVAKISMPVNTP(G)		598.648+++	23	6
X	X	(K)ISMPVNTPGQFEDFFPASSR(D)		743.005+++	55	6,7,8,9
X	X	(R)DQSSYLQGFSR(N)		644.311	75	2
X	X	(R)NESSYLQGFSR(N)	N for D (1), E for Q (2)	644.311	75	2
X	X	(R)NTLEAAFNAEFNEIR(R)		869.906	86	1,3,4,5,6,7,8,9,10
X	X	(F)NEIRRVLVQENAGGEQEERG(Q)	V for I (14)	1141.539	23	6
X	X	(R)VLLEENAGGEQEER(G)		786.885	74	2,4,6,8,10
X		(R)SSENNEGVIVK(V)		588.294	85	5,6,7,9,10
X		(S)SENNEGVIVKVS(K)		690.867	23	5, 6
X		(S)ENNEGVIVKVSKEHVQE(L)	Q for E (16)	721.692+++	27	7
X		(K)EHVEELTK(H)		492.753	82	1,2,5,6,7
	X	(K)EHVQELTK(H)		492.273	87	3
X		(A)KSISKKGSEEEGDITNPI(N)	I for V (3)	734.971+++	27	4
X		(S)KKGSEEEGDITNPINLREGE(P)		758.387+++	27	5
X		(K)KGSEEEGDITNPINLR(E)		591.321+++	40	6,7
	X	(K)GSEEEDITNPINLR(D)		793.881	79	7
X		(R)EGEPDLSNNFGK(L)		653.821	77	4, 5, 7 8,9
X	X	(K)LFEVKPDDK(N)		552.332	78	3,4,5,6,7
X	X	(K)EGALMLPHFNSK(A)		448.580+++	49	4,5,6,7,9
X	X	(A)LVIVVVNKGTGNLEL(V)	L for M (1)	591.316+++	22	9
X	X	(K)AMVIVVVNK(G)		486.807	63	3
X	X	(A)MMIVVVNKGTGNLELV(A)	M for V (2)	887.950	24	7
X	X	(K)GTGNLELVAVR(K)		564.811	71	1,4,6,7
X		(R)EEEEDEDEEEEGSNR(E)		912.858	41	5,6
X	X	(S)SRKVR(R)	S for N (1), K for E (3)	330.203	100	1,4
X	X	(S)SRQVR(R)	S for N (1), Q for E (3)	330.169	100	1,3,5,6,7,8,9
X	X	(A)AHPIAVNASSELHLL(G)	I for V(4), V for I (6)	592.289+++	28	10
X	X	(V)AINASSELHV(L)	V for L (10)	1037.485+	29	5
X	X	(L)GFGINAENNHR(I)		614.804	65	3,4,5,6,7,10
X	X	(R)IFLAGDKDNVIDQVEK(Q)		606.662+++	53	9,10
X	X	(Q)AEDLAFPGSGEQVEKMIENQ(R)		548.753++++	32	9
X	X	(K)DNVVDQIEKQAK(D)	V for I (4)	887.932	23	2
X	X	(K)DLAFPGSGEQVEK(L)		688.829	37	1,3,4,5,6,7
X	X	(G)SGEQVEKLIENQ(K)	E for K (10)	512.582+++	25	4
X	X	(K)GPLLSIMKAFN(-)	M for L (7)	477.581+++	27	1,3,4,5,8

```
isoform 1          1 MRGRVSPLMLLLGILVLASVSATHAKSSPYQKKTENPCAQRCLQSCQQEPDDLKQKACES  60
isoform 2          1 MRGRVSPLMLLLGILVLASVSATQAKS-PY-RKT ENPCAQRCLQSCQQEPDDLKQKACES 58

isoform 1         61 RCTKLEYDPRCVYDPRGHTGTTNQRSPPGERTRGRQPGDYDDDRRQPRREEGGRWGPAGP 120
isoform 2         59 RCTKLEYDPRCVYD----TGATNQRHPPGERTRGRQPGDYDDDRRQPRREEGGRWGPAEP 114

isoform 1        121 REREREEDWRQPREDWRRPSHQQPRKIRPEGREGEQEWGTPGSHVREETSRNNPFYFPSR 180
isoform 2        115 REREREEDWRQPREDWRRPSHQQPRKIRPEGREGEQEWGTPGSEVREETSRNNPFYFPSR 174

isoform 1        181 RFSTRYGNQNGRIRVLQRFDQRSRQFQNLQNHRIVQIEAKPNTLVLPKHADADNILVIQQ 240
isoform 2        175 RFSTRYGNQNGRIRVLQRFDQRSKQFQNLQNHRIVQIEARPNTLVLPKHADADNILVIQQ 234

isoform 1        241 GQATVTVANGNNRKSFNLDEGHALRIPSGFISYILNRHDNQNLRVAKISMPVNTPGQFED 300
isoform 2        235 GQATVTVANGNNRKSFNLDEGHALRIPSGFISYILNRHDNQNLRVAKISMPVNTPGQFED 294

isoform 1        301 FFPASSRDQSSYLQGFSRNTLEAAFNAEFNEIRRVLLEENAGGEQEERGQRRWSTRSSEN 360
isoform 2        295 FFPASSRDQSSYLQGFSRNTLEAAFNAEFNEIRRVLLEENAGGEQEERGQRRRSTRSSDN 354

isoform 1        361 NEGVIVKVSKEHVEELTKHAKSVSKKGSEEEGDITNPINLREGEPDLSNNFGKLFEVKPD 420
isoform 2        355 -EGVIVKVSKEHVQELTKHAKSVSKKGSEEE-DITNPINLRDGEPDLSNNFGRLFEVKPD 412

isoform 1        421 KKNPQLQDLDMMLTCVEIKEGALMLPHFNSKAMVIVVVNKGTGNLELVAVRKEQQQRGRR 480
isoform 2        413 KKNPQLQDLDMMLTCVEIKEGALMLPHFNSKAMVIVVVNKGTGNLELVAVRKEQQQRGRR 472

isoform 1        481 E---EEEDEDEEEEGSNREVRRYTARLKEGDVFIMPAAHPVAINASSELHLLGFGINAEN 537
isoform 2        473 EQEWEEEEEDEEEEGSNREVRRYTARLKEGDVFIMPAAHPVAINASSELHLLGFGINAEN 532

isoform 1        538 NHRIFLAGDKDNVIDQIEKQAKDLAFPGSGEQVEKLIKNQKESHFVSARPQSQSQSPSSP 597
isoform 2        533 NHRIFLAGDKDNVIDQIEKQAKDLAFPGSGEQVEKLIKNQRESHFVSARPQSQSPS---- 588

isoform 1        598 EKESPEKEDQEEENQGGKGPLLSILKAFN 626
isoform 2        589 ---SPEKEDQEEENQGGKGPLLSILKAFN 614
```

Fig. 4 – Aligned sequence of two isoforms of the peanut Allergen Ara h 1 and peptides that were identified by Q-TOF MS/MS. Identified peptides are highlighted on a grey background and isoform-specific peptides are highlighted in dark grey. The cleaved-off N-terminal peptide is indicated with italics. Underlined residues overlap with know IgE epitopes.

and purified allergens as well as antibody-reactive proteins were individually subjected to mass spectrometry identification (spot numbers in Figs. 1–3). The MS/MS spectra that were obtained were subjected to a search against databases to identify peptides by means of their fragment masses.

Table 1 lists the results of the analysed spots and the proteins that were identified by MS/MS. Ara h 1 was identified in spots 1–10, Ara h 2 in spots 20 and 22, Ara h 3/4 in spots 11 to 13 as well as in spot 17 (spot numbering as indicated in Figs. 1, 2a and 3). In spots 14 to 16 and 18 to 24 no peanut-specific peptides were found, but several residual peptides with high score originating from digested trypsin were identified, which indicates that both trypsin digestion and MS/MS analysis were successful.

a

```
isoform 1          1 MAKLTILVALALFLLAAHASARQQWELQGDRRCQSQLERANLRPCEQHLMQKIQRDEDSY 60
isoform 2          1 MAKLTILVALALFLLAAHASARQQWELQGDRRCQSQLERANLRPCEQHLMQKIQRDEDSY 60

isoform 1         61 ERDPYSPSQDPYSPS------------PYDRRGAGSSQHQERCCNELNEFENNQRCMCEA 108
isoform 2         61 GRDPYSPSQDPYSPSQDPDRRDPYSPSPYDRRGAGSSQHQERCCNELNEFENNQRCMCEA 120

isoform 1        109 LQQIMENQSDRLQGRQQEQQFKRELRNLPQQCGLRAPQRCDLDVESGG---- 156
isoform 2        121 LQQIMENQSDRLQGRQQEQQFKRELRNLPQQCGLRAPQRCDLEVESGGRDRY 172
```

b

```
isoform 1          1 MAKLTILVALALFLLAAHASARQQWELQGDRRCQSQLERANLRPCEQHLMQKIQRDEDSY 60
isoform 2          1 MAKLTILVALALFLLAAHASARQQWELQGDRRCQSQLERANLRPCEQHLMQKIQRDEDSY 60

isoform 1         61 ERDPYSPSQDPYSPS------------PYDRRGAGSSQHQERCCNELNEFENNQRCMCEA 108
isoform 2         61 GRDPYSPSQDPYSPSQDPDRRDPYSPSPYDRRGAGSSQHQERCCNELNEFENNQRCMCEA 120

isoform 1        109 LQQIMENQSDRLQGRQQEQQFKRELRNLPQQCGLRAPQRCDLDVESGG---- 156
isoform 2        121 LQQIMENQSDRLQGRQQEQQFKRELRNLPQQCGLRAPQRCDLEVESGGRDRY 172

isoform 1 = Allergen Ara h2.01
isoform 2 = Allergen Ara h2.02
```

Fig. 5 – Aligned sequence of two isoforms of the peanut Allergen Ara h 2 and peptides that were identified by Q-TOF MS/MS. Identified peptides for a) the purified peanut allergen Ara h 2, and b) for individual spots of 2D gels of peanut protein extracts (spots numbered 20 and 22 in Figs. 1–3) are highlighted on a grey background and isoform-specific peptides are highlighted in dark grey. The cleaved-off N-terminal peptide is indicated with italics.

In the case of allergen Ara h 1, the databank search using the mass of the fragments ions identified peptide sequences that are listed in Table 2. Forty five and 37 distinct peptides consisting of 5–22 amino acids were identified for the two known isoforms of Ara h 1 (Ara h 1 clone P41B and Ara h 1 clone P17 respectively numbered isoforms 1 and 2 in Table 2). Fig. 4 shows the alignment of the two known sequences of Ara h 1 and the peptides identified by MS/MS. Identified tryptic peptides listed in Table 2 were either specific (highlighted in dark grey) to a single isoform of Ara h 1 or are present in both and correspond to the conserved part of the sequence. Previously, we have reported the detection and identification of several peptides from Ara h 1 in peanut extracts that differ for one or two amino acids and are characteristic for either of the particular isoforms [35]. In the current study employing 2D gels, several peptides are unique for isoform 1 or isoform 2 (peptides in grey colour and corresponding spot IDs in Table 2). Based on specific peptide detection, the protein in spots 2, 4, 8 and 9 was identified as isoform 1 (protein probability of 100% in Table 1). Spot 3 only contains isoform 2 with a protein probability of 100% (Table 1) while spots 5–7 were identified as containing both distinct isoforms. Based on the detected peptides the software identified both isoforms in those spots and returned a protein probability of 100% for both. Also spots 1 and 10 are likely to contain both isoforms, but here the identification is based on a protein probability of ca. 50% (Table 1).

The identification of immunologically relevant epitopes as reported by Shin *et al.* [21], Bannon and Ogawa [41] and Cong *et al.* [42] allows a more complete analysis of the allergen derived peptide sequence tags identified in this study. Several peptides overlap completely or partially with IgE binding epitopes of the peanut allergen Ara h 1 (underlined residues in Fig. 4).

Wichers *et al.* [23] identified isolated proteins by 1D SDS-PAGE as Ara h 1 isoforms by N-terminal amino acid sequencing. They identified a protein band at ca. 64 kDa which was shown to contain a mixture of both isoforms. However, the first 78 and 84 amino acids of the cloned sequences, Swiss-Prot entries P43238 (isoform 1) and P43237 (isoform 2) respectively, appeared to be missing. In our work, the modified peptide, VASISATHAKSSPYQKKTENPC (residues 17–38 of isoform 1, variants underlined, 2 amino acid substitutions, peptide ladder score 22), likely to correspond to the N-terminal part of Ara h 1, is detected in spot 5 (Fig. 3). It corresponds to a validated peptide and overlaps partially with identified IgE epitopes identified by Shin *et al.* [21] and Cong *et al.* [42]. This peptide was found in the three consecutive replicate runs and therefore can be accepted with a confident identification.

The spots numbered 19 to 22 were investigated repeatedly to confirm the presence of the second major allergen Ara h 2 by MS measurements. However, the analyses after trypsin hydrolysis did not return any result, which is likely due to its trypsin inhibitory properties that were highlighted

Table 3 – List of identified peptides derived from the peanut allergen Ara h 2.

Protein Name: **Allergen Ara h2.01** — Protein MW (Da): 19755, pI: 5.2 — Source and accession number: SwissProt/TrEMBL, Q941R0 isoform 1

Protein Name: **Allergen Ara h 2.02** — Protein MW (Da): 20114, pI: 5.2 — Source and accession number: SwissProt/TrEMBL, Q6PSU2 isoform 2

A)

Isoform 1	Isoform 2	Peptide sequence	Substitution	Submitted mass (Da)$^{a)}$	**Peptide ladder score (%)$^{b)}$
X	X	(W)ELQGDR(R)		359,195	51
X	X	(R)ANLRPC(E)		365,694	60
X	X	(R)ANLRPCEQ(H)		494,252	68
X	X	(R)ANLRPCEQH(L)		375,525+++	35
X	X	(A)NLKPCEQ(H)	K for R (3)	444,733	48
X		(R)DEDSYERDPY(S)		644,768	63
	X	(P)HSPSQDPD(R)	H for Y (1)	448,195	31
X	X	(L)NEFENNQR(C)		525,747	64
X	X	(I)MENQSDRL(Q)		496,741	44
X	X	(M)ENQSDRL(Q)		431,220	35
	X	(C)DLEVESGGRD(R)		538,763	40
	X	(C)DLEVESGGRDR(Y)		411,546+++	30

B)

Isoform 1	Isoform 2	Peptide sequence	Substitution	Submitted mass (Da)$^{a)}$	**Peptide ladder score (%)$^{b)}$	Spot number in gel
X	X	(L)NEFENNQR(C)		525,745	55	20,22
X	X	(R)QQEQQFKR(E)		364,514	49	20,22

A) shows Ara h 2 derived peptides identified for the purified allergen Ara h 2 and B) peptides identified in individual spots of 2D gels of peanut protein extracts (spots numbered 20 and 22 in Figs. 1–3). Reported sequences are Ara h 2.01 and Ara h 2.02 (source: Swiss-Prot, entry numbers Q941R0 and Q8GV20). Peptides on a dark grey background are specific for one of the two isoforms of Ara h 2. a) Double charged peptide unless otherwise indicated (+, +++ or ++++). b) Peptide ladder score: probability based score algorithm, which indicates the reliability of the peptide identification.

Table 4 – List of identified peptides derived from the peanut allergen Ara h 3/4.

Protein Name: **Ara h 3 Glycinin** [Fragment]	Protein MW (Da): 58314, pI: 5.88	Source and accession number: SwissProt/TrEMBL, O82580	isoform 1
Protein Name: **Gly 1**	Protein MW (Da): 60448, pI: 5.48	Source and accession number: SwissProt/TrEMBL, Q9FZ11	isoform 2
Protein Name: **Ara h 4**	Protein MW (Da): 60973, pI: 5.35	Source and accession number: SwissProt/TrEMBL, Q9SQH7	isoform 3
Protein Name: **Allergen Ara h 3 / Ara h 4**	Protein MW (Da): 61738, pI: 5.39	Source and accession number: SwissProt/TrEMBL, Q8LKN1	isoform 4
Protein Name: **Glycinin** [Fragment]	Protein MW (Da): 58061, pI: 5.41	Source and accession number: SwissProt/TrEMBL, Q6IWG5	isoform 5
Protein Name: **Iso-Ara h 3**	Protein MW (Da): 58227, pI: 5.27	Source and accession number: SwissProt/TrEMBL, Q0GM57	isoform 6

Isoform						Sub-unit[a)]	Peptide sequence	Substitution	Submitted mass (Da)[b)]	Peptide ladder score (%)[c)]	Spot number in gel
1	2	3	4	5	6						
X	X	X	X	X	X	A	(R)LNAQRPDNR(I)		361.844+++	49	11,12,13
X	X	X	X			A	(Y)SNAPQEIFIQQGR(G)		744.381	47	11,12,13
			X			A	(R)FQGQDQSQQQQDSHQK(V)		639.597+++	26	13
			X	X	X	A	(E)GDLIAMPTGVAFWMYND(H)	M for V (6)	638.958+++	15	12
				X	X	A	(E)NKGSN(I)	K for E (2)	519.213+	56	11,12,13
X	X					A	(R)QIVQNLR(G)		435.763	81	11,12,13
				X	X	A	(R)QTVENLR(G)		430.239	36	11
X						A	(R)GETESEEEGAIVTVR(G)		795.867	25	11
			X			A	(R)GENESDEQGAIVTVR(G)		802.376	49	13
	X					A	(R)GENESEEEGAIVTVK(G)		795.867	80	12
	X	X		X	X	B	(T)ICTASV(K)		316.664	60	12
X	X	X	X			B	(L)NLMILQ(W)	M for L (3), Q for R (6)	759.359+	72	12
			X			B	(R)AHVQVVDSNGDR(V)		432.881+++	30	17
X			X	X	X	B	(F)KTDSRPSIANLAG(E)		759.354	36	17

The table shows Ara h 3/4 derived peptides identified in individual spots of 2D gels of peanut protein extracts (spots numbered 11–13 and 17 in Figs. 1–3). Reported sequences were Ara h 3 Glycinin, Gly1, Ara h 4, Allergen Ara h 3/Ara h 4, Glycinin and Iso-Ara h 3 (source: Swiss-Prot, entry numbers O82580, Q9FZ11, Q9SHQH7, Q8LKN1, Q6IWG5 and Q0GM57). Peptides on a dark grey background are not present in all isoforms of the allergen Ara h 3/4 (isoforms numbered 1–6).

a) A = acidic sub-unit.
B = basic sub-unit (according to Piersma *et al.* [14]).

b) Double charged peptide unless otherwise indicated (+ or +++).

c) Peptide ladder score: probability based score algorithm, which indicates the reliability of the peptide identification.

previously. Sequence homology searches have revealed that Ara h 2 has significant homology with trypsin inhibitors and bifunctional trypsin/α-amylase inhibitors. The trypsin inhibitory properties of purified Ara h 2 were also shown in enzyme activity assays [40]. Although we previously identified Ara h 2 derived peptides in unfractionated peanut protein extracts [35], the observation that partial reduction of this allergen increases its trypsin inhibitory activity [40]

```
isoform 1     1     -----------------------RQQPEENACQFQRLNAQRPDNRIESEGGYIETWNPN 36
isoform 2     1     MIRGRLALSVCFCFLVLGASSISFRQQPEENACQFQRLNAQRPDNRLESEGGYIETWNPN 60
isoform 3     1     -MAKLLELSFCFCFLVLGASSISFRQQPEENACQFQRLNAQRPDNRIESEGGYIETWNPN 59
isoform 4     1     -MGKLLALSVCFCFLVLGASSISFRQQPEENACQFQRLNAQRPDNRIESEGGYIETWNPN 59
isoform 5     1     ---KLLALSLCFCVLVLGASSVTFRQGGEENECQFQRLNAQRPDNRIESEGGYIETWNPN 57
isoform 6     1     -MAKLLALSLCFCVLVLGASSVTFRQGGEENECQFQRLNAQRPDNRIESEGGYIETWNPN 59

isoform 1     37    NQEFECAGVALSRLVLRRNALRRPFYSNAPQEIFIQQGRGYFGLIFPGCPRHYEEPHTQG 96
isoform 2     61    NQEFECAGVALSRLVLRRNALRRPFYSNAPQEIFIQQGRGYFGLIFPGCPSTYEEPAQQG 120
isoform 3     60    NQEFECAGVALSRLVLRRNALRRPFYSNAPQEIFIQQGRGYFGLIFPGCPSTYEEPAQQG 119
isoform 4     60    NQEFECAGVALSRLVLRRNALRRPFYSNAPQEIFIQQGRGYFGLIFPGCPSTYEEPAQQG 119
isoform 5     58    NQEFQCAGVALSRTVLRRNALRRPFYSNAPLEIYVQQGSGYFGLIFPGCPSTYEEPAQEG 117
isoform 6     60    NQEFQCAGVALSRTVLRRNALRRPFYSNAPLEIYVQQGSGYFGLIFPGCPSTYEEPAQEG 119

isoform 1     97    RRSQSQRPPRRLQ-GEDQSQQQR-DSHQKVHRFDEGDLIAVPTGVAFWLYNDHDTDVVAV 154
isoform 2     121   RRHQSQRAPRRFE-GEDQSQQQQDSHQKVRRFDEGDLIAVPTGVALWMFNDHDTDVVAV 179
isoform 3     120   RRYQSQRPPRRLQ-EEDQSQQQ-DSHQKVHRFNEGDLIAVPTGVAFWLYNDHDTDVVAV 177
isoform 4     120   RRHQSQRPPRRFQ-GQDQSQQQ-DSHQKVHRFDEGDLIAVPTGVAFWMYNDHDTDVVAV 177
isoform 5     118   RRYQSQKPSRRFQVGQDDPSQQQQDSHQKVHRFDEGDLIAVPTGVAFWMYNDEDTDVVTV 177
isoform 6     120   RRYQSQKPSRRFQVGQDDPSQQQQDSHQKVHRFDEGDLIAVPTGVAFWMYNDEDTDVVTV 179

isoform 1     155   SLTDTNNNDNQLDQFPRRFNLAGNTEQEFLRYQQQSRQSRRRSLPYSPYSPQSQPRQEER 214
isoform 2     180   SLTDTNNNDNQLDQFPRRFNLAGNHEQEFLRYQQQS---RRRSLPYSPYSPQSQPRQEER 236
isoform 3     178   SLTDTNNNDNQLDQFPRRFNLAGNHEQEFLRYQQQSRQSRRRSLPYSPYSPHSRPRREER 237
isoform 4     178   SLTDTNNNDNQLDQFPRRFNLAGNHEQEFLRYQQQS---RRRSLPYSPYSPQTQPKQEDR 234
isoform 5     178   TLSDTSSIHNQLDQFPRRFYLAGNQEQEFLRYQQQQ--------------GSRP--HYR 220
isoform 6     180   TLSDTSSIHNQLDQFPRRFYLAGNQEQEFLRYQQQQ--------------GSRP--HYR 222

isoform 1     215   EFSPRGQHSRRERAGQEEENEGGNIFSGFTPEFLEQAFQVDDRQIVQNLRGETESEEEGA 274
isoform 2     237   EFSPRGQHSRRERAGQEEENEGGNIFSGFTPEFLAQAFQVDDRQIVQNLRGENESEEEGA 296
isoform 3     238   EFRPRGQHSRRERAGQEEEDEGGNIFSGFTPEFLEQAFQVDDRQIVQNLWGENESEEEGA 297
isoform 4     235   EFSPRGQHGRRERAGQEQENEGGNIFSGFTPEFLAQAFQVDDRQILQNLRGENESDEQGA 294
isoform 5     221   QISPR-------VRGDEQENEGSNIFSGFAQEFLQHAFQVD-RQTVENLRGENEREEQGA 272
isoform 6     223   QISPR-------VRGDEQENEGSNIFSGFAQEFLQHAFQVD-RQTVENLRGENEREEQGA 274

isoform 1     275   IVTVRGGLRILSPDRKRR-----ADEEEEYDEDEYEYDEE----DRRRGRGSRGRGNGIE 325
isoform 2     297   IVTVKGGLRILSPDRKRG-----ADEEEEYDEDEYEYDEE----DRRRGRGSRGRGNGIE 347
isoform 3     298   IVTVRGGLRILSPDGTRG-----ADEEEEYDEDQYEYHEQ----DGRRGRGSRGGGNGIE 348
isoform 4     295   IVTVRGGLRILSPDRKRRQQYERPDEEEEYDEDEYEYDEEERQQDRRRGRGSRGSGNGIE 354
isoform 5     273   IVTVKGGLRILSPDEEDESSRSPPSRREEFDEDRSRPQQR-----GKYDENRRGYKNGIE 327
isoform 6     275   IVTVKGGLRILSPDEEDESSRSPPSRREEFDEDRSRPQQR-----GKYDENRRGYKNGIE 329

isoform 1     326   ETICTASAKKNIGRNRSPDIYNPQAGSLKTAND--LNLLILRWLGPSAEYGNLYRNALFV 383
isoform 2     348   ETICTASVKKNIGRNRSPDIYNPQAGSLKTAND--LNLLILRWLGLSAEYGNLYRNALFV 405
isoform 3     349   ETICTACVKKNIGGNRSPHIYDPQRWFTQNCHD--LNLLILRWLGLSAEYGNLYRNALFV 406
isoform 4     355   ETICTASFKKNIGRNRSPDIYNPQAGSLKTANELQLNLLILRWLGLSAEYGNLYRNALFV 414
isoform 5     328   ETICSASVKKNLGRSSNPDIYNPQAGSLRSVNE--LDLPILGWLGLSAQHGTIYRNAMFV 385
isoform 6     330   ETICSASVKKNLGRSSNPDIYNPQAGSLRSVNE--LDLPILGWLGLSAQHGTIYRNAMFV 387

isoform 1     384   AHYNTNAHSIIYRLRGRAHVQVVDSNGNRVYDEELQEGHVLVVPQNFAVAGKSQSENFEY 443
isoform 2     406   PHYNTNAHSIIYALRGRAHVQVVDSNGNRVYDEELQEGHVLVVPQNFAVAGKSQSDNFEY 465
isoform 3     407   PHYNTNAHSIIYALRGRAHVQVVDSNGNRVYDEELQEGHVLVVPQNFAVAGKSQSENFEY 466
isoform 4     415   PHYNTNAHSIIYALRGRAHVQVVDSNGDRVFDEELQEGHVLVVPQNFAVAGKSQSENFEY 474
isoform 5     386   PHYTLNAHTIVVALNGRAHVQVVDSNGNRVYDEELQEGHVLVVPQNFAVAAKAQSENYEY 445
isoform 6     388   PHYTLNAHTIVVALNGRAHVQVVDSNGNRVYDEELQEGHVLVVPQNFAVAAKAQSENYEY 447

isoform 1     444   VAFKTDSRPSIANLAGENSVIDNLPEEVVANSYGLQREQARQLKNNNPFKFFVPPS-QQS 502
isoform 2     466   VAFKTDSRPNIANFAGENSIIDNLPEEVVANSYGLPREQARQLKNNNPFKFFVPPS-QQS 524
isoform 3     467   VAFKTDSRPSIANFAGENSFIDNLPEEVVANSYGLPREQARQLKNNNPFKFFVPPF-QQS 525
isoform 4     475   VAFKTDSRPSIANLAGENSFIDNLPEEVVANSYGLPREQARQLKNNNPFKFFVPPS-EQS 533
isoform 5     446   LAFKTDSRPSIANLAGENSIIDNLPEEVVANSYRLPREQARQLKNNNPFKFFVPPFDHQS 505
isoform 6     448   LAFKTDSRPSIANLAGENSIIDNLPEEVVANSYRLPREQARQLKNNNPFKFFVPPFDHQS 507

isoform 1     503   PRAVA 507
isoform 2     525   LRAVA 529
isoform 3     526   PRAVA 530
isoform 4     534   LRAVA 538
isoform 5     506   MREVA 510
isoform 6     508   MREVA 512
```

Fig. 6 – Aligned sequence of six isoforms of the peanut Allergen Ara h 3/4 and peptides that were identified by Q-TOF MS/MS. Identified peptides are highlighted on a grey background and isoform-specific peptides are highlighted in dark grey. The cleaved-off N-terminal peptide is indicated with italics. The N-termini of the acidic and the basic sub-unit are shown in bold. Underlined residues overlap with know IgE epitopes.

might explain why MS identification after SDS-PAGE was unsuccessful.

Investigations for in-solution enzymatic hydrolysis of the purified allergen Ara h 2 have been carried out. Various enzymes were tested and among them pancreatin returned positive results. MS experiments return several tags for Ara h 2 (12 peptides returned, 6–11 amino acids). The two sequences of Ara h 2 were aligned and are represented in Fig. 5. Eight sequence tags were common to Ara h 2.01 and Ara h 2.02 isoforms (Fig. 5a and Table 3A). The presence of the two distinguished isoforms, namely Ara h 2.01 and Ara h 2.02, as mentioned by Chatel *et al.* [24] and Hales *et al.* [25], was confirmed. One peptide was shown to be specific to Ara h 2.01 and two peptides to Ara h 2.02 (Fig. 5a and Table 3A). Selected peptides had a score over 20 and were validated. In the same way, Ara h 2 individual spots (numbered 19–22) were treated according to optimised conditions on the purified allergens. MS experiments returned two sequence tags after in-gel digestion of spot numbers 20 and 22 (2 unmodified peptides, 8 amino acids, peptide ladder scores of 49 and 55) (Fig. 5b and Table 3B). Spots 19 and 21 did not return results and they may correspond to glycosylated forms of the protein. Ara h 2 was unambiguously identified in spots 20 and 22, but the peptides detected are located in the conserved part of the protein and did not allow to distinguish between the two isoforms.

In the case of the allergen Ara h 3/4, database searches successfully identified several sequence tags from four spots, numbered 11 to 13 and 17 (Table 1). From the Swiss-Prot database, the following sequences were retrieved for the allergen Ara h 3/4: Ara h 3 Glycinin, Gly1, Ara h 4, Allergen Ara h 3/Ara h 4, Glycinin and Iso-Ara h 3, numbered isoforms 1–6 in Table 4. A total of 14 different peptides consisting of 5–17 amino acids were experimentally identified for Ara h 3/4. Among the peptides found five were specific either to isoform 1, 2 or 4 (peptides coloured in dark grey in Table 4).

Multiple sequences of Ara h 3/4 were aligned and are represented in Fig. 6. Peptides identified by MS/MS analysis are highlighted on a grey background and specific peptides are highlighted in dark grey. The analysis of immunologically relevant epitopes of the allergen Ara h 3/4 was reported by Rabjohn *et al.* [12] and Bannon and Ogawa [41]. Two of the peptides identified in our study partially overlap with an IgE binding epitope (underlined residues in Fig. 6). Piersma *et al.* [14] indicated the presence of at least five isoforms of Ara h 3/4 in a single peanut variety. In our work, the large number of highly homologous peptides prevented the assignment of spots to single isoforms. However, spots could be assigned to different parts of the protein. In spots 11 and 12 (Fig. 3) two peptides were found that are specific for the isoforms 2 and 5. In addition to this, in both spots several peptides of the conserved part of Ara h 3/4 sequences were found. According to Piersma *et al.* [14], RQQPEEN (residues 1–7 of isoform 1) corresponds to the N-terminus of the acidic sub-unit and GIEETIC (residues 323–329 of isoform 1) corresponds to the N-terminus of the basic sub-unit of the allergen Ara h 3/4 (N-termini shown in bold in Fig. 6). Peptides originating from the three spots numbered 11 to 13 (at ca. 40–45 kDa and a pI range of 5.5–6) mostly correspond to conserved parts of the protein and are located close to its N-terminal end. This part corresponds to the acidic sub-unit of Ara h 3/4. In addition, in spot 12 (mass of ca. 45 kDa and a pI of ca. 5.7) two peptides corresponding to the conserved part of the basic sub-unit were found. However, both are located far from the C-terminus of Ara h 3/4 (Table 4 and Fig. 6). The occurrence of Ara h 3/4 that is not cleaved at the boundary of the basic and acidic sub-unit was reported previously with the detection of the peptide NGIEETICTASAK (residues 322–334 of isoform 1) that partially covers both sub-units [35]. On the other hand, spot 17 (mass of ca. 25 kDa and a pI of ca. 6.3) contains the basic sub-unit only. Two peptides located close to the C-terminal end of Ara h 3/4 were identified with high peptide ladder scores. Spots 23 and 24 recognised by the IgY antibody raised against the recombinant 40 kDa sub-unit of Ara h 3/4 (Figs. 2d and 3) are likely to be proteolytic fragments of the acidic sub-unit. However, they were only defined in MS by small peptides (up to 5 amino acids) and therefore assignment to a protein sequence could not be done.

Our results are in agreement with Piersma *et al.* [14] who established MALDI-MS and LC-MS/MS spectra of the tryptic digest of protein bands excised after 1D SDS-PAGE. This revealed Ara h 3/4 protein bands at 25, 40, 42 and 45 kDa. The basic sub-unit at ca. 25 kDa appeared essentially as a single polypeptide, while for the acidic sub-unit three bands, ranging from 40–45 kDa, were observed and identified. In addition, multiple minor bands ranging from 14–20 kDa were identified as peptides originating from the acidic sub-unit, indicating proteolytic truncation of the protein in the peanut. Sequence variants were observed in both the acidic and basic sub-unit indicating the presence of at least five Ara h 3/4 genes for a single peanut variety.

Liang *et al.* [36] and Magni *et al.* [43] used 2D PAGE to achieve a better resolution of protein profiles of peanut seeds. Based on peptide sequencing by ESI-MS/MS and N-terminal amino acid sequences, Liang *et al.* [36] identified 4 protein spots in the range 23–25 kDa with distinct isoelectric points (range 3.9–6.8); all peptide fragments had significant homology with the known isoforms 1, 3 and 6 and essentially correspond to the basic sub-unit. Our study confirms the complexity of Ara h 3/4 with the identification of several proteolytic fragments of this allergen and the detection of multiple isoforms.

4. Concluding remarks

In conclusion, the novelty of this work lies in the development of an integrated approach using complementary techniques. The combination of 2D DIGE, Western blotting and LC Q-TOF MS/MS allows the specific detection and identification of major allergens and has revealed their position within 2D gel maps. Within this map the allergen Ara h 1 was resolved and detected in 10 protein spots. The identification of all 10 spots was achieved by spiking with purified allergen, Western blotting and MS/MS. The position of Ara h 2 allergens, that are known to inhibit trypsin activity, was established by spiking and Western blotting detecting 4 and 2 protein spots respectively. MS/MS analyses identified two Ara h 2 derived peptides in 2 of the 4 spots.

The 2D pattern of the purified Ara h 3/4 appears to be more complex and matched to 8 spots, four of which were recognised by anti Ara h 3 antibodies raised against a fragment of the allergen. MS/MS analyses identified a multitude of Ara h 3/4 derived peptides in four protein spots, and confirmed the presence of multiple isoforms in a single peanut variety. The identification by MS/MS allowed different spots to be assigned to different parts of this protein, which is known to be proteolytically processed.

The identification of a multitude of isoforms and protein sub-units of the major peanut allergens stresses the complexity of the peanut allergens. The establishment of their specific location within the 2D gel map provides us important tools to further investigate the complexity of post-translational allergen modification by for instance glycosylation and glycation. The characterisation of individual peanut allergens and their resolution into different isoforms, glycoforms and proteolytic products is of importance to study and quantify the effect of various thermal treatments such as roasting on peanut allergens. The identification and characterisation of peanut allergens is also essential to understand exactly which compounds are presented to the immune system.

Acknowledgment

The authors are grateful to Dr. Franz Ulberth for valuable comments on the manuscript.

REFERENCES

[1] Ortolani C, Ipsano M, Scibilia J, Pastorello EA. Introducing chemists to food allergy. Allergy 2001;56:5–8.
[2] Woods RK, Stoney RM, Raven J, Walters EH, Abramson M, Thien FC. Reported adverse food reactions overestimate true food allergy in the community. Eur J Clin Nutr 2002;56:31–6.
[3] Mills ENC, Jenkins JA, Alcocer MJC, Shewry PR. Structural, biological, and evolutionary relationships of plant food allergens sensitizing via the gastrointestinal tract. CRC Rev Food Sci Nutr 2004;44:379–407.
[4] European Parliament and Council. Directive 2000/13/EC of 20 March 2000 on the approximation of the laws of the Member States relating to the labelling, presentation and advertising of foodstuffs. Official J Europ Commun 2000;L109:29–42.
[5] European Parliament and Council. Directive 2003/89/EC of 10 November 2003 amending Directive 2000/13/EC as regards indication of the ingredients present in foodstuffs. Official J Europ Union 2003;L308:15–8.
[6] European Commission Directive 2007/68/EC of 27 November 2007 amending Annex IIIa to Directive 2000/13/EC of the European Parliament and of the Council as regards certain food ingredients. Official J Europ Union 2007;L310:11–4.
[7] Sampson HA, McCaskill CC. Food hypersensitivity and atopic dermatitis: evaluation of 113 patients. J Pediatr 1985;107:669–75.
[8] Koppelman SJ, Bruijnzeel-Koomen CADM, Hessing M. Heat induced conformational changes of Ara h 1, a major peanut allergen, do not affect its allergenic properties. J Biol Chem 1999;274:4770–7.
[9] Dunwell JM. Cupins: a new superfamily of functionally-diverse proteins that include germins and plant seed storage proteins. Biotechnol Genet Eng Rev 1998;15:1–32.
[10] Pomes A, Helm R, Bannon G, Burks A, Tsay A, Chapman M. Monitoring peanut allergen in food products by measuring Ara h 1. J Allergy Clin Immunol 2005;111:641–5.
[11] Viquez OM, Summer CG, Dodo HW. Isolation and molecular characterization of the first genomic clone of a major peanut allergen, Ara h 2. J Allergy Clin Immunol 2001;107:713–7.
[12] Rabjohn P, Helm EM, Stanley JS, West CM, Sampson HA, Burks AW, et al. Molecular cloning and epitope analysis of the peanut allergen Ara h 3. J Clin Invest 1999;103:535–42.
[13] Viquez OM, Konan N, Dodo H. Genomic characterisation of the third major peanut allergen gene Ara h3/4. Proceedings of the American Peanut Research & Educational Society (APRES), vol. S94; 2002. p. 19.
[14] Piersma SR, Gaspari M, Helfe SL, Koppelman SJ. Proteolytic processing of the peanut allergen Ara h 3. Mol Nutr Food Res 2005;49:744–55.
[15] Kleber-Janke T, Crameri R, Appenzeller U, Schlaak M, Becker WM. Selective cloning of peanut allergens, including profilin and 2S albumins, by phage display technology. Int Arch Allergy Immunol 1999;119:265–74.
[16] Boldt A, Fortunato D, Conti A, Petersen A. Analysis of the composition of an immunoglobulin E reactive high molecular weight protein complex of peanut extract containing Ara h 1 and Ara h 3/4. Proteomics 2005;5:675–86.
[17] Burks AW, Sampson HA, Bannon GA. Peanut allergens. Allergy 1998;53:725–30.
[18] Koppelman SJ, Vlooswijk RAA, Knippels LMJ, Hessing M, Knol EF, van Reijsen FC, et al. Quantification of major peanut allergens Ara h 1 and Ara h 2 in the peanut varieties Runner, Spanish, Virginia and Valencia, bred in different parts of the world. Allergy 2001;56:132–7.
[19] Burks AW, Williams LW, Helm RM, Caunnaughton C, Cockrell G, O'Brien TJ. Identification of a major peanut allergen, Ara h 1, in patients with atopic dermatitis and positive peanut challenges. J Allergy Clin Immunol 1991;88:172–9.
[20] Buschmann L, Petersen A, Schlaak M, Becker WM. Reinvestigation of the major peanut allergen Ara h 1 on molecular level. Monogr Allergy 1996;32:92–8.
[21] Shin DS, Compadre CM, Maleki SJ, Kopper RA, Sampson H, Huang SK, et al. Biochemical and structural analysis of the IgE binding sites on ara h1, an abundant and highly allergenic peanut protein. J Biol Chem 1998;273:13753–9.
[22] Burks AW, Cockrell G, Stanley JS, Helm RM, Bannon GA. Recombinant peanut allergen Ara h I expression and IgE binding in patients with peanut hypersensitivity. J Clin Invest 1995;96:1715–21.
[23] Wichers HJ, De Beijer T, Savelkoul HFJ, van Amerongen A. The major peanut allergen Ara h 1 and its cleaved-off N-terminal peptide; possible implications for peanut allergen detection. J Agric Food Chem 2004;52:4903–7.
[24] Chatel JM, Bernard H, Orson FM. Isolation and characterisation of two complete Ara h 2 isoforms cDNA. Int Arch Allergy Immunol 2003;131:14–8.
[25] Hales BJ, Bosco A, Mills KL, Hazell LA, Loh R, Holt PG, et al. Isoforms of the major peanut allergen ara h 2: IgE binding in children with peanut allergy. Int Arch Allergy Immunol 2004;135:101–7.
[26] Koppelman SJ, Knol EF, Vlooswijk RAA, Wensing M, Knulst AC, Helfe SL, et al. Peanut allergens Ara h 3: isolation from peanut and biochemical characterisation. Allergy 2003;58:1144–51.
[27] Restani P, Ballabio C, Corsini E, Fiocchi A, Isoardi P, Magni C, et al. Identification of the basic subunit of Ara h 3 as the major allergen in a group of children allergic to peanuts. Ann Allergy Asthma Immun 2005;94:262–6.
[28] Jung R, Scott MP, Nam YW, Beaman TW. The role of proteolysis in the processing and assembly of 11s see globulins. Plant Cell 1998;10:343–57.
[29] Hanash SM. Biomedical applications of two-dimensional electrophoresis using immobilized pH gradients: current status. Electrophoresis 2000;21:1202–9.

[30] Goerg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, et al. The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 2000;21:1037–105.
[31] Chassaigne H, Brohée M, Nørgaard JV, van Hengel AJ. Investigation on sequential extraction of peanut allergens for subsequent analysis by ELISA and 2D gel electrophoresis. Food Chem 2007;105:1671–81.
[32] Tonge R, Shaw J, Middleton B, Rowlinson R, Rayner S, Young J, et al. Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology. Proteomics 2001;1:377–96.
[33] González-Buitrago JM, Ferreira L, Isidoro-García M, Sanz C, Lorente F, Dávila I. Proteomic approaches for identifying new allergens and diagnosing allergic diseases. Clin Chim Acta 2007;385:21–7.
[34] Sen M, Kopper R, Pons L, Abraham EC, Burks AW, Bannon GA. Protein structure plays a critical role in peanut allergen stability and may determine immunodominant IgE-binding epitopes. J Immunol 2002;169:882–7.
[35] Chassaigne H, Nørgaard JV, van Hengel AJ. Proteomics-based approach to detect and identify major allergens in processed peanuts by capillary HPLC-Q-TOF (MS/MS). J Agric Food Chem 2007;55:4461–73.
[36] Liang XQ, Luo M, Holbrook CC, Guo BZ. Storage protein profiles in Spanish and runner market type peanuts and potential markers. BMC Plant Biol 2006;6:1–9.
[37] Burks AW, Williams LW, Caunnaughton C, Cockrell G, O'Brien TJ, Helm RM. Identification and characterisation of a second major allergen, Ara h 2, with use of the sera of patients with atopic dermatisis and positive peanut challenge. J Allergy Clin Immunol 1992;90:962–9.
[38] Burks AW, Helm RM. Major peanut allergen Ara h 2. 1996, US Patent 5558869.
[39] Schein CH, Ivanciuc O, Braun W. Common physical–chemical properties correlate with similar structure of the IgE epitopes of peanut allergens. J Agric Food Chem 2005;53:8752–9.
[40] Maleki SJ, Viquez O, Jacks T, Dodo H, Champagne ET, Chung S-Y, et al. The major peanut allergen, Ara h 2, functions as a trypsin inhibitor, and roasting enhances this function. J Allergy Clin Immunol 2003;112:190–5.
[41] Bannon GA, Ogawa T. Evaluation of available IgE-binding epitope data and its utility in bioinformatics. Mol Nutr Food Res 2006;50:638–44.
[42] Cong Y, Lou F, Xue W, Li L, Chena M. Characterisation of the IgE-binding immunodominant epitopes on Ara h1. Food Agric Immunol 2008;19:175–85.
[43] Magni C, Ballabio C, Restani P, Sironi E, Scarafoni A. Two-dimensional electrophoresis and western-blotting analyses with anti Ara h 3 basic subunit IgG evidence the cross-reacting polypeptides of *Arachis hypogaea*, *Glycine max* and *Lupinus albus* seed proteomes. J Agric Food Chem 2005;53:2275–81.